

Microenvironmental changes by placenta-derived mesenchymal stem cells restore the ovarian function in ovariectomized rat via activated PI3K-FOXO3 pathway

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Research

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Abstract

Background: Translational studies have explored the therapeutic potential and feasibility of mesenchymal stem cells (MSCs) in several degenerative diseases; however, the mechanistic studies of the function of these cells have been insufficient. As ovarian failures cause anovulation as well as ovarian steroid hormonal unbalances, the specific aims of this study were to analyze the therapeutic role of placenta derived MSCs (PD-MSCs) in an ovarian-failure ovariectomy (OVX) rat model and evaluate whether PD-MSCs transplantation (Tx) improved folliculogenesis and oocyte maturation in the injured ovary through PI3K/Akt and FOXO signaling.

Methods: Blood and ovary tissue were collected and analyzed after various PD-MSCs Tx treatments in the ovariectomized rat model. Changes in the expression of folliculogenesis and ovary regeneration-related genes due to PD-MSCs treatments were analyzed by qRT-PCR, Western blotting, and histological analysis.

Results: The levels of hormones related to ovary function were significantly increased in the PD-MSCs Tx groups compared with those of the non-transplantation group (NTx). The follicle numbers in the ovarian tissues were increased along with increased expression of genes related to folliculogenesis for PD-MSCs Tx compared with NTx groups. Furthermore, PD-MSCs Tx induced maturation of follicles by increasing the phosphorylation of GSK3 beta and FOXO3 ($p < 0.05$) and shifting the balance of growth and apoptosis in the oocytes.

Conclusions: Taken together, PD-MSCs Tx can restore the ovarian function as well as induce ovarian folliculogenesis via the PI3K/Akt and FOXO signaling pathway.

Background

Ovarian failure is characterized by the premature loss of ovary follicles and is a common disease in premenopausal women receiving treatment chemotherapy or radiation. Ovarian dysfunction in women can have serious medical consequences as blood clots, heart disease and osteoporosis as well as infertility [1]. For causes other than chemotherapy or radiation, polycystic ovary syndrome (PCOS) in particular is a common female pathology that affects 5~10% of women of reproductive age. They are infertile, because of aberrant follicle growth by immature follicles [2]. These irregularities in folliculogenesis are further defined by an abnormal relationship between growth of oocyte and the surrounding granulosa cells [3] in terms of several hormones including anti-Mullerian hormone (AMH), FSH and estrogen. The most common treatment for the induction of ovulation is hormone replacement therapy; however, it can increase the risk for ovarian and breast cancer [4, 5].

Mesenchymal stem cells (MSCs) have been in the spotlight as a promising cell source to treat a variety of degenerative diseases because their self-renewal activity, the potential to differentiate to a variety of different cell types and their immunomodulatory effects. Recently, Johnsons et al. showed that BM-MSCs transplantation restored oocyte production in mice lacking ovulation as they had been previously treated by chemotherapy [6]. In addition, BM-MSCs improved their ovarian structure and function via production

of VEGF, IGF-1 and HGF in the rats with chemotherapy-induced ovarian damage and it had the potential to generate immature oocytes and enable long-term fertility in mouse model treated with chemotherapy [7, 8]. However, there is some controversy on the exact role of BM-MSCs affecting ovarian dysfunction. According to Santiquet et al., transplanted BM-MSCs did not generate new oocytes in a mice model, although the fertility of female SCID mice post-chemotherapy was improved after transplantation with the bone marrow derived cells [9]. As another cell source, it has been demonstrated that human amniotic fluid stem cells (hAFCs) have the potential to differentiate to follicle oocytes as well as having the therapeutic effect for restoring ovarian function in mice with chemotherapy-induced sterility [10, 11]. Mesenchymal stem cells derived from the human placenta, namely human placenta-derived mesenchymal stem cells (PD-MSCs), also have several advantages: 1) there is no donor age-dependence as most cells are of early stage in development; 2) these cells have high proliferation activity; 3) the cells are easily accessible; 4) they are abundant; and 5) they are strongly immunosuppressive compared to other mesenchymal stem cells derived from the bone marrow or adipose tissues [12, 13]. Also, we reported that PD-MSCs highly expressed HLA-G compared to other MSCs including BM and adipose MSCs as well as the PD-MSCs suppressed the activation of T-cell in an *in vitro* co-culture system [14]. Because of these immunosuppressive properties, there are ongoing clinical trials with PD-MSCs in several disease types including certain autoimmune diseases [15-17]. Recently, Yin N et al., showed that PD-MSCs have therapeutic benefits in mice induced with premature ovarian failure by regulating regulatory T-cell activity and cytokines [18, 19].

However, as the mechanisms for PD-MSCs therapeutic effect have not been fully addressed, we investigated whether PD-MSCs might induce expression of genes related to ovarian regeneration and whether they retained the ability to restore ovarian function in a rat model with ovarian failure according to various transplantation routes. Furthermore, we scrutinized the signaling mechanisms by which transplanted PD-MSCs activated and were involved in ovarian regeneration in a rat model of ovariectomy.

Materials And Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC 1500072) of the CHA laboratory animal research center at Sampyeong-dong in Gyeonggi, Korea. Female Sprague-Dawley rats (Orient Corporation, Seongnam, Gyeonggi, Korea) that were 8-weeks-old were used in this study. All rats were housed at two per pathogen-free cage at room temperature (21°C) with 12 hours light-dark cycle, given ad libitum access to water and standard commercial food.

Isolation and culture of chorionic-plate mesenchymal stem cells

The collection of human placenta and their use were approved by the Institutional Review Board (IRB) of CHA General Hospital, Seoul, Korea (IRB07-18). Placentas were collected from women who delivered at term (38±2 gestational weeks). Placenta-derived mesenchymal stem cells were isolated from normal chorionic plate of term placentas, as previously described [20]. PD-MSCs were cultured in DMEM/F12

medium supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 U/ml; Thermo Fisher Scientific), streptomycin (100 µg/ml; Thermo Fisher Scientific), fibroblast growth factors 4 (25 ng/ml; Peprotech, Rocky Hill, NJ), and 1 µg /ml heparin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C under 5% CO₂.

Generation of ovarian failure model by ovariectomy and PD-MSC transplantation into a rat model

Following acclimatization, the rats were randomly divided into three groups. The NTx group, ovariectomized (OVX) rats (n=25); the DTx group, directly PD-MSCs transplanted OVX-rats through ovary (n=45); the TTx group, indirectly PD-MSCs transplanted OVX-rats through tail vein injection (n=45). Ovariectomy was performed in rats of all groups to remove one of ovaries. All rats were anesthetized by intraperitoneal injection with 250 mg/kg Avertin (Sigma-Aldrich). The surgical site (pelvic area of the back) was disinfected with ethanol, and then, through the skin and muscle incision of 1~2 cm, one ovary was accessed and removed by excision. After removal of the ovary, the incision was sutured and was disinfected with povidone-iodine (Sigma-Aldrich). One week after the ovariectomy, PD-MSCs were stained using a PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich) and injected through the remaining ovary (DTx; 1 x 10⁵ cells) or tail vein (TTx; 5 x 10⁵ cells). Non-transplanted rats were injected with culture medium. Blood samples were collected weekly and measured for plasma E₂ using an Estradiol DSL-4400 Radioimmunoassay Kit (Diagnostic System Laboratories, Inc, Webster, TX, USA) before sacrifice. Rats were sacrificed and ovary tissues were harvested at 1, 2, 3 and 5 weeks.

Ex vivo culturing of the ovary

PD-MSCs were plated in 24-well culture plates at density of 1x10⁴ cells per well, cultured in modified-culture medium [α -MEM (Thermo Fisher Scientific) containing 2% FBS (Thermo Fisher Scientific), 1X penicillin/streptomycin (Thermo Fisher Scientific), 25 ng/ml FGF4 (Peprotech), and 1 µg /ml heparin (Sigma-Aldrich)] at 37°C under condition of 5% CO₂. After 24 hours, the inserts were pre-coated with matrigel (BD Biosciences, San Jose, CA, USA) for 3 hours and fitted on each well of 24-well plate containing PD-MSCs or only culture medium. The ovary tissues isolated from the normal rats were cut into half size using a sterile scalpel blade (JEUNGDO Bio and Plant Co., Ltd, Seoul, Korea) and placed in the upper chamber of the matrigel-coated insert. Co-culture plates were incubated in 37°C incubator at 5% CO₂ for 24, 48 and 72 hours.

Hormone ELISA assays

Blood samples were collected from rats of NTx, DTx, and TTx groups via the retro-orbital technique. Serum was separated from whole blood by using a serum separating tube (SST; BD Biosciences). Both the serum samples and the supernatants from the ex-vivo cultures were analyzed using ELISA kits for the anti-Mullerian hormone (AMH; Elabscience, Beijing, China), and the follicle-stimulating hormone (FSH, Abbexa, Cambridge, UK), according to the manufacturer's instructions. Briefly, an equal volume of a given sample was added to the specific-antibody labeled plates. Then, specific HRP-conjugates were added to

each well, and incubated. Next, the substrates were added to each well and incubated in dark. After the substrate development, the activity of the antibodies was analyzed using a microplate reader (BioTek, Winooski, VT). Concentration of Estradiol (E2) was measured by a chemiluminescence immunoassay (CLIA) using UniCel-Dxl800 auto immunoassay analyzer (Beckman-Coulter, Brea, CA, USA).

Genomic DNA isolation

Genomic DNA (gDNA) was extracted from the rat ovaries by treatment with proteinase K (QIAGEN, Valencia, CA, USA) and phenol/chloroform (Sigma-Aldrich). This involved the ovary tissues of rats ground in LN2, followed by tissue powders digested in the digestion buffer [100 mM Tris pH 8.0 (Abelbio, Jungnang, Seoul, Korea) buffer containing 5 mM EDTA (Bioneer, Daedeok, Dajeon, Korea), 0.2% sodium dodecyl sulfate (SDS; Bioneer), 200 mM sodium chloride (Bioneer), and 0.5 mg/ml proteinase K (Dako, Cambridge, U.K)] at 55°C and incubation overnight. The supernatants containing gDNA were extracted by using phenol/chloroform (1:1, Sigma-Aldrich), and damped down by isoamyl alcohol (Sigma-Aldrich) and 0.3 M sodium acetate (Bioneer) at -20°C overnight. Next, the pellet of gDNA was washed with 70% cold ethanol and was eluted using Tris-EDTA buffer. The gDNA for each sample were analyzed using 1% agarose gel electrophoresis.

RNA isolation and quantitative real-time polymerase chain reaction analysis

Total RNA was homogenized and extracted from rat ovary using a Trizol reagent (Invitrogen Thermo Fisher, Camarillo, CA USA). Total RNA was reversely transcribed into cDNA using Superscript III RNase H reverse transcriptase (Invitrogen) and according to the manufacturer's protocols. Briefly, first cDNA transcription of total RNA (500 ng) was performed with oligo dT (Invitrogen), and dNTP mix (Invitrogen) at 65°C for 5 minutes followed by second cDNA synthesis performed at 50°C for an hour and at 72°C for 15 minutes with DTT (Invitrogen), RNase out (Invitrogen), Superscript III (Invitrogen), RNase H (Invitrogen) and reverse transcriptase (Invitrogen). cDNA and gDNA were amplified with specifically designed primers (Table S1) and detected using A SYBR Green master mix (Roche Diagnostics, Basel, Switzerland) and Exicycler™ 96 PCR system (Bioneer). The cDNA amplification conditions for qRT-PCR were denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 5 seconds, and 59°C for 30 seconds. All experiments were performed in triplicate. The expression levels were calculated using the Ct method after normalization to mRNA levels of GAPDH as an internal control.

Western blots

Rat ovary tissues harvested from the animal experiments or the ex-vivo cultures were ground, sonicated, and lysed in protein lysis buffer [RIPA buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors (A.G. Scientific, San Diego, CA, USA)]. Total protein extracts (45 µg) were separated on 8%~15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad, Hercules, CA, USA). The membranes were blocked with 8% skim milk or 5% BSA (Amresco, Solon, OH, USA) for 1 hour at room temperature (RT), and then incubated with primary antibodies (1:1000) at 4°C overnight, followed by secondary antibodies

(1:20,000) for 1 hour at RT using an orbital shaker. The expression was detected with an ECL Advanced Western Blot Detection kit (Amersham, Marlborough, MA, USA) and by ChemiDoc™ XRS+ System (Bio-Rad). Antibodies used in this study included anti-rabbit-Nanos3 (Abcam, Cambridge, UK), anti-mouse-Nobox (Santa Cruz Biotechnology, Dallas, TX, USA), anti-goat-LHX8 (Santa Cruz), anti-rabbit-p-Akt(Ser473) (Cell Signaling, Danvers, MA, USA), anti-rabbit-t-Akt (Cell Signaling), anti-rabbit-PI3K (Cell Signaling), anti-rabbit-p-GSK3beta (Cell Signaling), anti-rabbit-p-FOXO3alpha (Cell Signaling), anti-rabbit-caspase9 (Abcam), anti-rabbit-caspase3 (BD Biosciences), anti-rabbit-GAPDH (Invitrogen), HRP-conjugated goat-anti-mouse IgG (Bio-Rad), HRP-conjugated donkey-anti-goat IgG (Sigma-Aldrich), anti-mouse and HRP-conjugated donkey-anti-rabbit IgG (Amersham). All experiments were performed in triplicate. Intensity of each band was quantified by Image J software (NIH, Bethesda, MD, USA)

Enzyme-linked immunosorbent assay (ELISA)

Both human and rat of Stem cell factor (SCF) and caspase-3 activities in ovarian tissue lysates and supernatants of ex-vivo culture were measured with the SCF ELISA (R&D Systems, Minneapolis, MN, USA) and Caspase-3 ELISA kits (Promega, Gangseo, Seoul, Korea), respectively, and according to the manufacturer's protocols. Also, cell necrosis was assessed by the release of lactate dehydrogenase (LDH) into the supernatants of the co-culture system. LDH activity was measured using a CytoTox 96 assay system (Promega) following the manufacturer's protocol. All experiments were performed in triplicate.

Hematoxylin and eosin (H&E) staining

To evaluate the number of follicles in rat ovary after ovariectomy and PD-MSC transplantation, H&E staining was performed. Rat ovary samples were processed in 10% formalin (Millipore, Billerica, MA, USA). Five-micron-thick paraffin sections at were stained with H&E, and visualized by inverted light microscope. The total number of follicles less than 100 µm in diameter was counted in at least ten selected non-overlapping fields.

Immunostaining

To analyze the expression and localization of LHX8 and Lin-28 in rat ovary following the transplantation of PD-MSCs, ovary samples were embedded in OCT compound (Fisher Scientific, Pittsburgh, PA, USA). Five-micron-thick cryostat sections were fixed in cold methanol for 10 min and permeabilized with proteinase K (Dako) for 5 min at RT. The sections were blocked by protein block serum-free buffer (Dako) at RT for 30 minutes, incubated first with anti-goat-LHX8 (1:100 dilutions, Santa Cruz) at 4°C overnight and next with anti-rabbit-Lin-28 (1:100 dilution, Abcam) in dark at RT for 2 hours. The mixture of secondary antibodies including Alexa Fluor 488 chicken anti-goat IgG (1:200 dilutions, Invitrogen) and Alexa Fluor 594 goat anti-rabbit IgG (1:200 dilution, Invitrogen) were incubated in antibody diluent (Dako) at RT for 1 hour followed by nuclei staining with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). The stained coverslips were mounted using mounting solution to avoid light loss. Images were visualized using an Olympus confocal microscope (x100 magnification) (Olympus, Tokyo, Japan <https://www.olympus-global.com>).

Statistical analysis

Data are represented as means and standard error (\pm SE). Significance values were calculated by Student's *t*-test and an ANOVA on SAS software (SAS Institute, Cary, NC, USA) and *p*-value of <0.05 was used to determine significance (labeled as * or #).

Results

PD-MSCs transplantation increased the levels of serum hormones in ovarian function

In order to investigate the effect of PD-MSCs on the hormone levels related to ovarian function depend on transplantation routes, we measured levels of estradiol (E2), anti-Mullerian hormone (AMH) and follicle-stimulating hormone (FSH) in serum of rats in the NTx, DTx and TTx groups at 1, 2, 3 and 5 weeks after PD-MSCs transplantation (Fig. 1). The levels of E2 were increased at 1 and 5 weeks after PD-MSCs transplantation comparing to those of NTx group for the DTx and TTx groups, respectively ($p < 0.05$); however, they were not significant at 2 and 3 weeks. In addition, the levels of E2 in the TTx group at 5 weeks were significantly higher than both the NTx and DTx groups ($p < 0.05$, Fig. 1a). Also, the production of AMH and FSH in the TTx group at 5 weeks was significantly higher compared to the other groups, respectively ($p < 0.05$, Fig. 1b and c). These data suggest the PD-MSCs transplantation can increase the hormones related to ovary function in ovariectomized (OVX) rats.

PD-MSCs engrafted into ovary increased the number of follicles

To confirm that the engraftment of PD-MSCs in a rat model with ovarian failure depends on transplantation routes, we analyzed the expression of human Alu gene as a human specific marker in the ovary tissues of rats using qRT-PCR. The expression of human Alu was not detected in the ovary of the NTx group, but was strongly expressed in the rat ovary for the DTx and TTx groups at 1, 2 weeks post-transplantation of PD-MSCs, although their expressions did fade out after 3 weeks post-transplantation of the PD-MSCs. Also, the expression of human Alu gene was higher in the DTx group than in the TTx group for 2 weeks post transplantation ($p < 0.05$, Fig. 2a). These results indicated that PD-MSCs were stably engrafted into the ovary tissues of in the rat model either by direct local transplantation or an intravenous injection.

We next analyzed whether engrafted PD-MSCs in the ovarian failure model improved the structure or the number of follicles in the ovary tissue of the rats. To quantify the number of follicles, the size of the follicles in an oocyte was defined as below 100 μ m and counted following H&E staining. The number of follicles in the ovary tissues in the DTx and TTx groups was significantly higher than the NTx group ($p < 0.05$, Fig. 2b and c). In addition, the number of follicles in ovary in the DTx group was highest at one week after transplantation, and the number of follicles in the TTx group was highest in 5 weeks post-transplantation ($p < 0.05$, Fig. 2b and c). These results suggest that PD-MSCs can dramatically increase the number of follicles in the OVX rat model in the TTx group; nevertheless, the efficiency of their

engraftment decreased after 3 weeks post-transplantation with the PD-MSCs for both the transplantation routes.

Expression of markers related to folliculogenesis by PD-MSCs were increased in the rat with OVX

It is well known that Nanos3, newborn ovary homeobox (Nobox) and LIM homeobox 8 (LHX8) are essential genes for folliculogenesis of mammalian ovary [21-23]. To evaluate whether PD-MSCs play a role in folliculogenesis in ovary via regulation of these genes, we analyzed expression of the above genes in the ovary tissue from OVX rats using qRT-PCR and Western blot. The mRNA message levels of Nanos3, Nobox and LHX8 in the ovary of rat model were significantly increased by PD-MSCs transplantation regardless of the transplantation route ($p < 0.05$, Fig. 3a). Their expressions were higher in the TTx group than those of the DTx group ($p < 0.05$, Fig. 3a). Similarly, the protein expression of Nanos3, Nobox and LHX8 in the TTx group was dramatically increased compared to the NTx and DTx groups; however, there were no differences the expressions of Nobox and LHX8 in the DTx group compared to the NTx group ($p < 0.05$, Fig. 3a and S1). Thus, PD-MSCs transplantation can promote the expression of folliculogenesis-related genes including Nanos, Nobox3 and LHX8 in the ovary of a rat model with OVX.

Increased LHX8 and Lin28a expressions in the ovary tissues after PD-MSCs transplantation involved in a balance of death and survival for oocytes by activation of the PI3k/Akt and Foxo3 pathway in a rat OVX model

Recently, Ren and colleagues reported that LHX8 was a critical oocyte-specific transcriptional factor for primordial follicle activation as well as postnatal folliculogenesis through LHX8-Lin28a interaction [24]. With this understanding, we checked whether the increased LHX8 expression by PD-MSCs transplantation affected the expression of Lin28a and whether the PI3K-Akt pathway was activated in the rat model with OVX. From our analysis, the mRNA expression of Lin28a was significantly increased in both DTx and TTx groups more than those of the NTx groups for all weeks post-transplantation, and in particular, the expression in the TTx group was higher than that in the DTx group ($p < 0.05$, Fig. 4a). Interestingly, the nuclear levels of oocyte Lin28 co-localizing with LHX-8 were higher after PD-MSCs transplantation in the ovarian follicles (Fig. 4b).

We also examined the effect of PD-MSCs on the balance of growth, survival and death in the oocytes in the ovary by looking at changes in the PI3k/Akt pathway. Expressions of PI3K and pAkt in the TTx group were dramatically increased compared to both NTx and DTx groups ($p < 0.05$, Fig. 4c and Fig. S2). In addition, the levels of well-known PI3K/Akt down-stream markers, pGSK3 β and pFOXO3a, were higher in the TTx group than in the NTx and DTx groups ($p < 0.05$, Fig. 4c, d and Fig. S2). On the other hand, representative levels of apoptosis markers including caspase-3 and caspase-9 were significantly decreased in the DTx and TTx groups compared to NTx group ($p < 0.05$, Fig. 4d and Fig. S2). These results indicate that transplanted PD-MSCs induce an increase in the expression of Lin28a and LHX8 and influence the balance between growth and death of oocytes in a rat model with OVX by activating the PI3K/Akt and FOXO3 pathway.

Ex vivo co-culture of PD-MSCs with ovarian tissues increases the AMH ovarian reserves, and this was not due to increased maturation of oocytes

In order to confirm the effect of PD-MSCs on hormones level produced by ovary, PD-MSCs was co-cultured with excised ovarian tissues. The ovarian tissues isolated from rats were co-cultured with PD-MSCs on pre-coated matrigel plates for up to 72 hours (Fig 5a). The levels of hormones including AMH and FSH in supernatants collected at 24, 48, and 72 hours after the co-culture were measured by ELISA. AMH, which is expressed in ovarian granulosa cells and serves as a biomarker for the relative size of the ovarian reserve, was significantly upregulated at 24 hours in the co-culture with the PD-MSCs than incubation with medium ($p < 0.05$, Fig. 5b). On the contrary, the levels of FSH, which stimulates the growth and recruitment of immature ovarian follicles in the ovary, were decreased ($p < 0.05$, Fig. 5c). There were no differences in the levels of AMH and FSH regardless of co-culturing with PD-MSCs or not at 72 hours and the levels of both these hormones decreased instead. These findings indicate that co-cultivated PD-MSCs induce an ovarian reserve in the excised ovarian tissues through stimulation of AMH produced by ovarian granulosa cells, not due to recruitment of immature ovarian follicles.

Co-culture of PD-MSCs induce folliculogenesis as well as growth of the ovary via increased stem cell factor ex vivo

Here, we demonstrated the increased mRNA expression of Nanos3, Nobox, LHX8 and Lin28a in the ovarian tissues co-cultured with PD-MSCs ex vivo; this is in line with transplanted PD-MSCs inducing the expression of several factors related to folliculogenesis in vivo in the rat model with OVX. Generally, the mRNA expressions of Nanos3, Nobox, LHX8 and Lin28a were more significantly increased at 48 hours, and the mRNA expressions of Nanos3 and Lin 28a being higher at 24 and 48 hours after co-culture with PD-MSCs compared to co-culture free groups ($p < 0.05$, Fig. 6a). This data further supports the previous results that the transplantation of PD-MSCs increases folliculogenesis through enhancing the expression of Nanos3, Nobox and LHX8. Similar to a positive effect on folliculogenesis, the levels of PI3K and pAkt at 24 or 48 hours were significantly increased, respectively, in the excised ovarian tissues after the co-culture with PD-MSCs compared to the co-culture free group ($p < 0.05$, Fig. 6a).

Furthermore, the levels of the PI3K/Akt down-stream markers, pFOXO3a and pGSK3 β , were significantly higher in the ovarian tissues at 48 hours after the co-culture with PD-MSCs than those with the co-culture free groups ($p < 0.05$, Fig. 6b and Fig. S3). The co-culturing with PD-MSCs reduced cell death from apoptosis (from active caspase-3 levels) and necrosis (LDH levels) in ovarian tissues compared to those of the co-culture free group, although the frequency of cell death by necrosis were increased for all ovarian tissues at 72 hours ($p < 0.05$, Fig. 6c). These findings indicate that PD-MSCs trigger activation of FOXO signaling in ovarian tissues resulting in induction of folliculogenesis as well as ovary growth. It is known that the interaction between Kit and stem cell factor (SCF, also known as Kit ligand) on granulosa cells triggers the activation of PI3K/Akt signaling pathway in ovarian tissues [25]. As such, we checked the expression of SCF in ovarian tissues co-cultured with PD-MSCs. The mRNA levels of SCF were significantly increased in ovarian tissues co-cultured with PD-MSCs compared to those of the co-culture

free and the co-culture with WI-38 human fibroblasts at 24 and 48 hours ($p < 0.05$, Fig. 6d, left panel). Also, the protein levels of SCF in the culture supernatants from co-cultured with PD-MSCs were significantly increased at 48 and 72 hours compared to those with the co-culture free group ($p < 0.05$, Fig. 6d, right panel).

Otherwise, their concentrations were decreased in the supernatant medium of co-culture with WI-38 cells as negative control (Fig. 6d). The concentration of human SCF in the culture supernatants from co-culture with PD-MSCs were significantly increased at 24 and 72 hours compared to those with the co-culture free groups ($p < 0.05$, Fig. S4). These findings indicate that co-culturing with PD-MSCs induced SCF expression in the excised ovarian tissue, and leading to activation of PI3K/Akt and FOXO3 signaling and reduction in levels of apoptosis.

Discussion

Ovarian failure can result from hereditary conditions or from exposure to various agents such as those used for cancer therapy. The factors that result in infertility are abnormal ovulation, anovulation (failure to ovulate), hyperandrogenism and metabolic abnormalities in women of childbearing age, manifesting in menopause, and causing various medical problems in the middle years of a woman's life [26]. Hormone replacement therapy (HRT) with recombinant hormones such as gonadotrophins to address ovarian failure, however, is often associated with side effects that include over-production of follicles with manifestations including the ovarian hyperstimulation syndrome and multiple pregnancies [27, 28]. To overcome these medical hurdles, various approaches are being developed, including cell-based therapy with stem cells for functional regeneration or enhancement of ovaries in ovarian failure cases. Recently, Takehara et al. showed that adipose-derived MSCs promoted the restoration of ovary function in an ovary failure model by decreasing the levels of inflammatory cytokines [29]. Wang et al. demonstrated that granulosa cells were affected by transplanted MSCs, as these cells helped restore folliculogenesis in the chemotherapy-induced ovarian failure mouse model [30]. Nevertheless, there are still ambiguities in therapeutic mechanisms concerning MSCs transplantation in ovarian failure models.

MSCs are attractive therapeutic agents, not only because of their potential for self-renewal and differentiation activities, but also for MSCs providing the proper microenvironment "niche" to enhance regeneration of cells or tissues damaged or cancer therapy through various signaling pathways and cellular events [31-33]. Therefore, diverse researches were tried to determine appropriate MSCs derived from various tissue by enhancing self-renewal, differentiation and anti-inflammation ability of MSCs, which is directly correlated with tissue regeneration [34, 35]. Recently, placenta-derived MSCs, which are a representative source of fetal stem cells, became regarded as an alternative to bone marrow-derived MSCs in potential medical applications. In pre-clinical studies with various animal models, PD-MSCs have already demonstrated a therapeutic potential for degenerative diseases and are currently being tested for such applications in clinical trials in worldwide. Further studies, however, on the fundamental mechanisms for their therapeutic efficacies are needed.

Previously, we demonstrated that PD-MSCs rapidly induced an increase in the levels of E2 hormone and led to changes in gene expression related to folliculogenesis in a rat model with ovariectomy when 3D spheroids of PD-MSCs were directly transplanted in the rats, although the precise mechanism of these transplanted cells were not known [36]. We next focused on identifying the most effective transplantation routes to maximize the therapeutic effect of PD-MSCs and investigated their effect on functional improvements including folliculogenesis and oocyte growth through the PI3K/Akt and pFOXO signaling in the rat model with ovariectomy. Interestingly, PD-MSCs engraftment by tail vein transplantation (TTx) showed a dramatic increase in the number of follicles until 5 weeks post-transplantation compared to other groups although their engraftment was not detected at over 2 weeks ($p < 0.05$, Fig. 2). The findings suggest that TTx is a safe and optimal transplantation route for PD-MSCs and engrafted PD-MSCs disappear in a short period after triggering folliculogenesis and changing the microenvironment in ovarian tissues in the rat model.

Alternation of the microenvironment niche by stem cells is one of the important factors required to understand the therapeutic mechanism of transplanted stem cells as well as enhancing their therapeutic effects [37]. In particular, in stem cell therapy, the cell-to-cell interactions and the cross-talk between stem cells and endogenous cells in the target tissues are critical in restoring and regenerating tissues in regenerative medicine [38]. In the present study, we confirmed that PD-MSCs transplantation induces an increase in the expression of folliculogenesis related genes (e.g., Nanos3, Nobox, and LHX8) including an RNA-binding protein, Lin28a, involved in regulation of many microRNAs [39, 40]. Lin28a blocks production of the mature let-7 microRNA in mouse embryonic stem cells through binding to the let-7 pre-microRNA and thus regulating the self-renewal activity of stem cells [41]. In addition, Lin28a is a well-known regulator of primordial germ cell development in human ovary development through the Akt/mTOR pathway [42]. Ren et al. also showed that LHX8 directly binds to Lin28a, resulting in increased Lin28a expression as it regulates primordial oocyte activation [24]. Our data indicates that PD-MSCs transplantation induces an increase in the expression of LHX8 as well as Lin28a in a rat model. These findings point to the increased LHX8/Lin28a signaling by PD-MSCs stimulating folliculogenesis through activation of PI3K/Akt signaling in the rat model with ovariectomy.

During mammalian oogenesis, the phosphatidylinositol-3-kinase (PI3K) signaling pathway is a critical regulator of survival, as the loss or activation of follicle development are regulated by the several components of the pathway including Akt, GSK3 α and GSK3 β [25], and in particular, PI3K-PTEN circuit governs follicle activation by contributing to the initiation of oocyte growth [43]. Recently, the oocyte-specific deletion of PTEN led to stimulation of PI3K activation of Akt phosphorylation and resulted in FOXO3 hyperphosphorylation and nuclear translocation in the oocytes, and led to stimulation of follicle activation [44]. Thus, PI3K pathway and FOXO3a may be important for oocyte growth as well as follicle activation. FOXO3, which is one of FOXO family members is related to female fertility by regulating the folliculogenesis both in oocytes and in somatic granulosa cells, and determines oocyte apoptosis or growth, and can lead to follicle activation [45].

The FOXO proteins have a "Janus face" for folliculogenesis according to their phosphorylated status and localization in the oocyte. For example, overexpression of FOXO3a in the oocyte nucleus can lead to apoptosis of the oocyte by upregulating caspase-3 and -8, the pro-apoptotic proteins, and Bim, FasL and p27KIP1, which are the key downstream mediators of them [46]. On the other hand, when FOXO3 is phosphorylated at an Akt site, it is exported from the nucleus to the cytoplasm, and results in follicle activation [44]. In spite of the importance of FOXO3a in ovarian function, the link between the therapeutic MSCs and modulation of FOXO3a localization and levels has not been reported. Based on the knowledge, we investigated the effect of PD-MSCs on growth and folliculogenesis in ovarian tissues in the rat model, and confirmed that PD-MSCs could restore the ovary function by promoting oocyte growth and inhibiting apoptosis in vivo and ex vivo in the co-culture system via the PI3K/Akt/FOXO signaling pathway. (Fig. 4 and 6).

In addition, the SCF-PI3K-Akt-FOXO3a mediated cascade by PD-MSCs may play an important role oocyte growth and follicular activation. In particular, SCF (Kit ligand; KL) produced by granulosa cells activates the PI3K pathway and serves as an intra-oocyte signal via the oocyte-surface receptor Kit [47]. In absence of SCF, interestingly, FOXO3a rather induces oocyte apoptosis, rather than oocyte growth, via the PI3K/Akt pathway, and thus, the SCF from the granulosa cells regulates the oocyte growth and follicular development by activating the PI3K-Akt-GSK β pathway in the oocytes [25, 48]. In a previous report, increased SCF by PD-MSCs activated PI3K expression, resulting in upregulation of PI3K-SCF-cKit signaling and promoting the self-renewal ability of PD-MSCs through mTOR phosphorylation under hypoxic conditions [49]. This implies that increased SCF by PD-MSCs can act as a signal for oocyte growth rather than cell death through PI3K/Akt/FOXO signaling and PD-MSCs modulate this pathway in restoring folliculogenesis and addressing ovarian dysfunction in a rat OVX model. This report is the first report of the molecular mechanism of therapeutic effect of PD-MSCs in restoring ovarian function in a rat model with OVX.

Conclusion

In summary, our results indicate that PD-MSCs transplantation can restore ovarian function by promoting folliculogenesis through upregulation of Nanos3, Nobox, LHX8 and Lin28a. The therapeutic effect could be accelerated by the SCF secreted from PD-MSCs capable of promoting oocyte survival by the PI3K/Akt/FOXO pathway. These results suggest that PD-MSCs can restore ovarian function by regulating the folliculogenesis and oocyte growth through the PI3K-FOXO3a pathway (Fig. 7). Our findings suggest that MSCs therapy using PD-MSCs can restore the ovarian function and be used as an alternative therapy for patients with ovarian dysfunction, including that for PCOS and infertility. In addition, this work offers new insights into a further understanding of MSCs-based therapeutic mechanisms in reproductive diseases.

Abbreviations

MSCs : Mesenchymal stem cells; PD-MSCs : Human placenta-derived mesenchymal stem cells; OVX : ovariectomy; GAPDH : glyceraldehyde-3-phosphate dehydrogenase; Nobox : Newborn ovary homeobox; LHX8 : LIM homeobox 8; PI3K : phosphoinositide 3 kinase; FOXO3 : forkhead box O3; DAPI : 4', 6-diamidino-2-phenylindole; AMH : anti-Mullerian hormone; FSH : follicle-stimulating hormone; LDH : lactate dehydrogenase; SCF : stem cell factor

Declarations

Acknowledgements

Not applicable.

Authors' contributions

J.H.C. did analysis and interpretation of data, and manuscript drafting. J.S. and S.M.L. did data analysis. T.H.K helped critical discussion. G.J.K. conceived and designed the experiments, and directed manuscript drafting, financial support and final approval of manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC 1500072) of the CHA laboratory animal research center at Sampyeong-dong in Gyeonggi, Korea.

Also, the collection of human placenta and their use were conducted under the guidelines and with the approval of the affiliated Institutional Review Board of the Gangnam CHA General Hospital, Seoul, Korea. (IRB 07-18). All patients consented to the respective use of their tissues.

Consent for publication: Not applicable.

Disclosure/conflict of interest

The authors declare no conflicts of interest.

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Figures

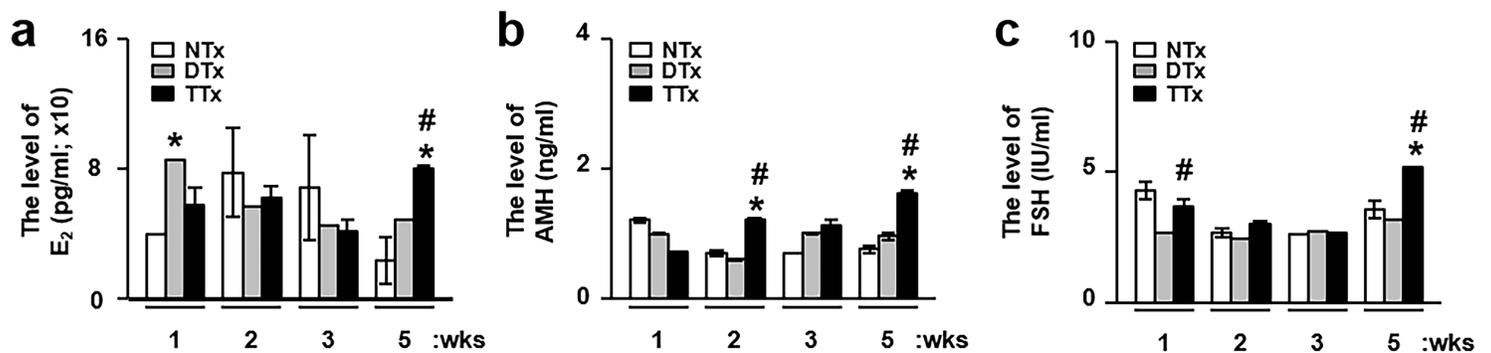


Figure 1

Levels of serum hormones in ovariectomized rats after PD-MSC transplantation. Hormone levels for (a) E₂, (b) AMH, and (c) FSH in the serum of ovariectomized rats at 1, 2, 3, and 5 weeks after PD-MSC

transplantation were analyzed by chemiluminescence immunoassays and enzyme-linked immunosorbent assays (mean±SE) (n=5 per group). All experiments were performed in triplicate. Significance at $p < 0.05$ is indicated by * for NTx vs. others and # for DTx vs. TTx.

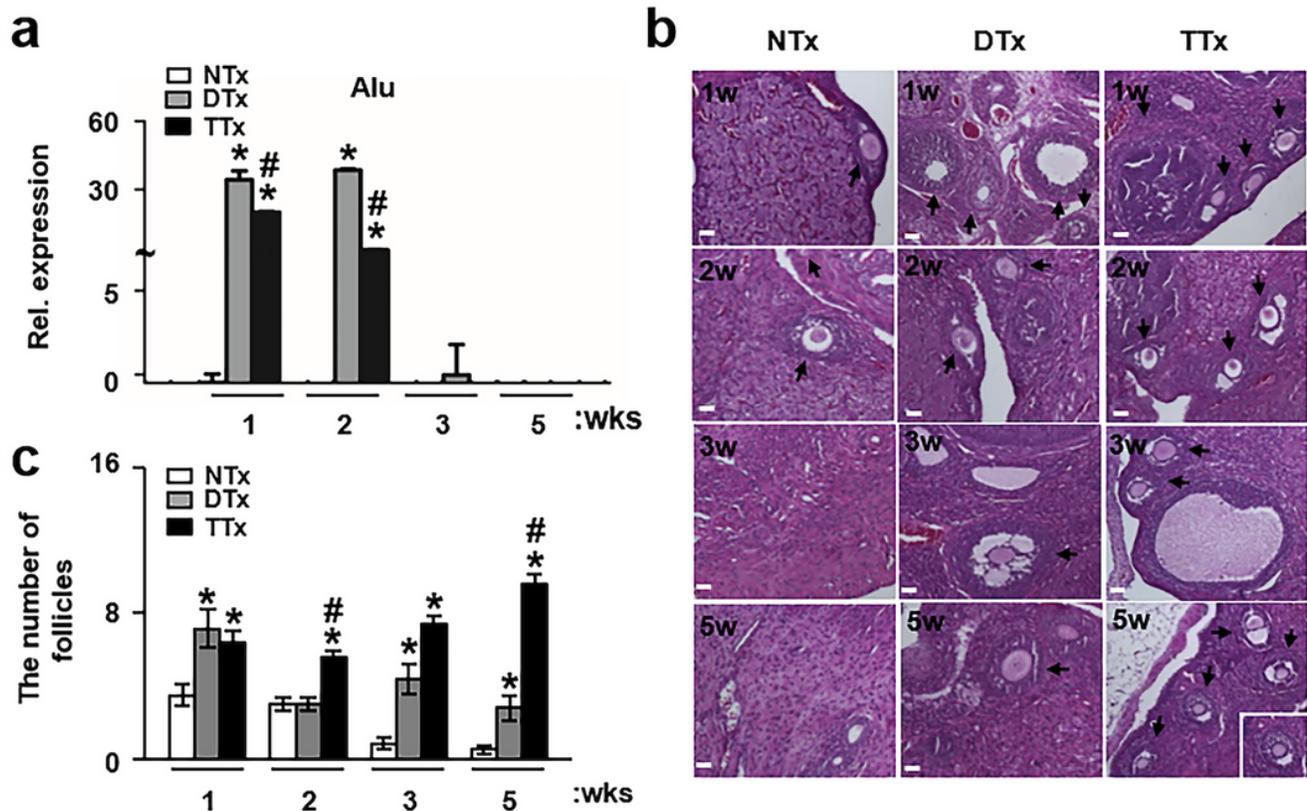


Figure 2

Number of follicles in the ovaries of rats depends on engraftment of PD-MSCs. (a) The expression of the human Alu gene in ovary tissues of ovariectomized rats at 1, 2, 3, and 5 weeks after PD-MSC transplantation was analyzed by quantitative real-time polymerase chain reaction analysis, normalized to GAPDH as an internal control (mean±SE) (n=5 per group). (b) Histological staining of ovaries in ovariectomized rats at 1, 2, 3, and 5 weeks after injection of PD-MSCs was performed with hematoxylin and eosin staining. Scale bars=50 μm. (c) The number of follicles ≤100 μm in the ovary was counted (mean±SE) (n=3 per group). Significance at $p < .05$ is indicated by * for NTx vs. others and # for DTx vs. TTx.

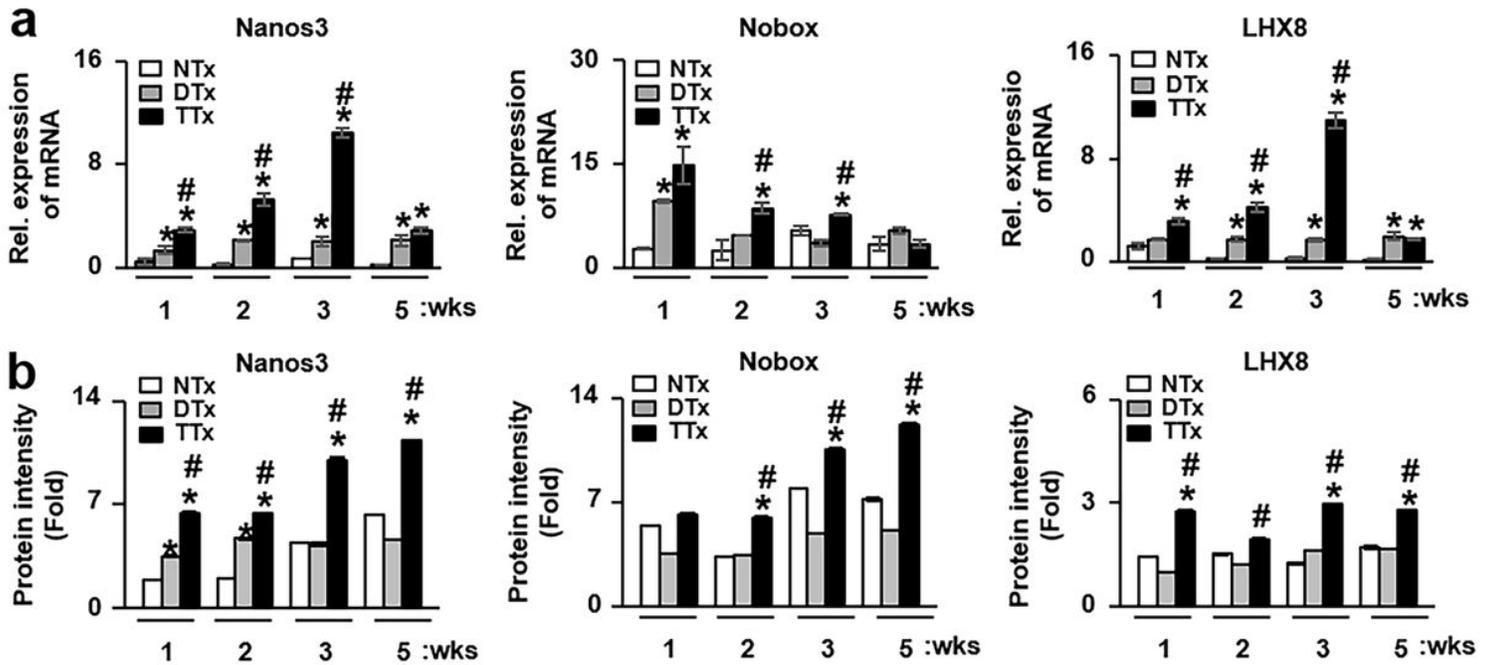


Figure 3

Expression of markers involved in ovarian function after PD-MSC transplantation. (a) The mRNA levels and (b) the protein levels of Nanos3, Nobox, and LHX8 in ovary tissue isolated from ovariectomized rats at 1, 2, 3, and 5 weeks after PD-MSC transplantation were measured by quantitative real-time polymerase chain reaction and Western blot (n=5 per group), respectively. GAPDH was used as a loading control (mean±SE). Significance at $p < 0.05$ is indicated by * for NTx vs. others and # for DTx vs. TTx. All experiments were performed in triplicate.

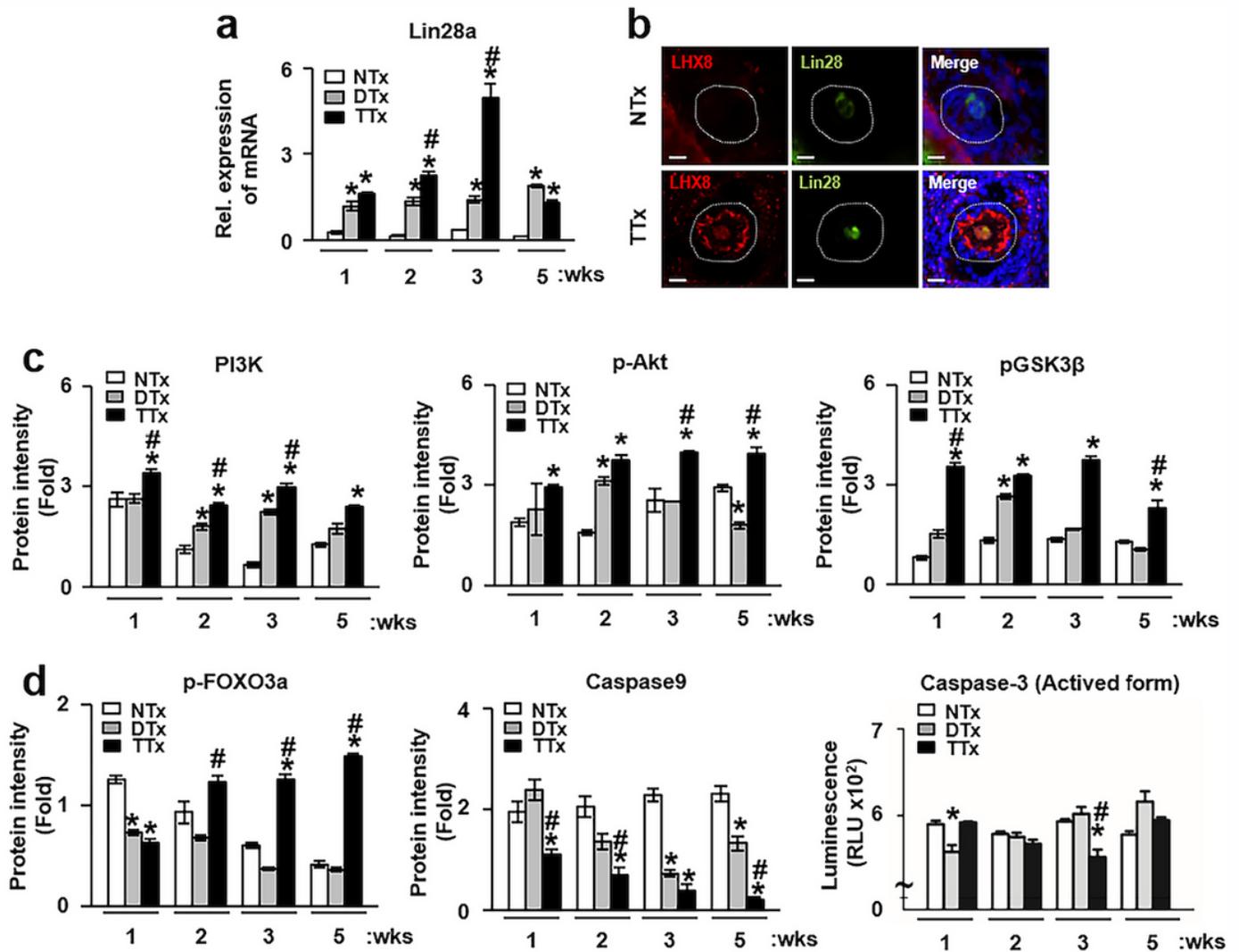


Figure 4

Expression of genes involved in ovarian function in the ovary after PD-MSC transplantation. (a) The expression of Lin28 mRNA in the ovaries of rats at 1, 2, 3, and 5 weeks after PD-MSC transplantation was analyzed by quantitative real-time polymerase chain reaction, with normalization to GAPDH as the internal control (mean±SE) (n=5 per group). (b) Localization of Lin28 (green) and LHX8 (red) was analyzed by immunofluorescence. Nuclei were stained with DAPI (blue). Scale bar: 50 μm. (c) The intensity of protein expression of PI3K, pAkt, pGSK3β, (d) FOXO3a, and caspase-9 and the activity of caspase-3 were analyzed by Western blotting and enzyme-linked immunosorbant assays and measured with ImageJ software (mean±SE) (n=5 per group). GAPDH was used as an internal control. All experiments were performed in triplicate. Significance at p <.05 is indicated by * for NTx vs. others and # for DTx vs. TTx.

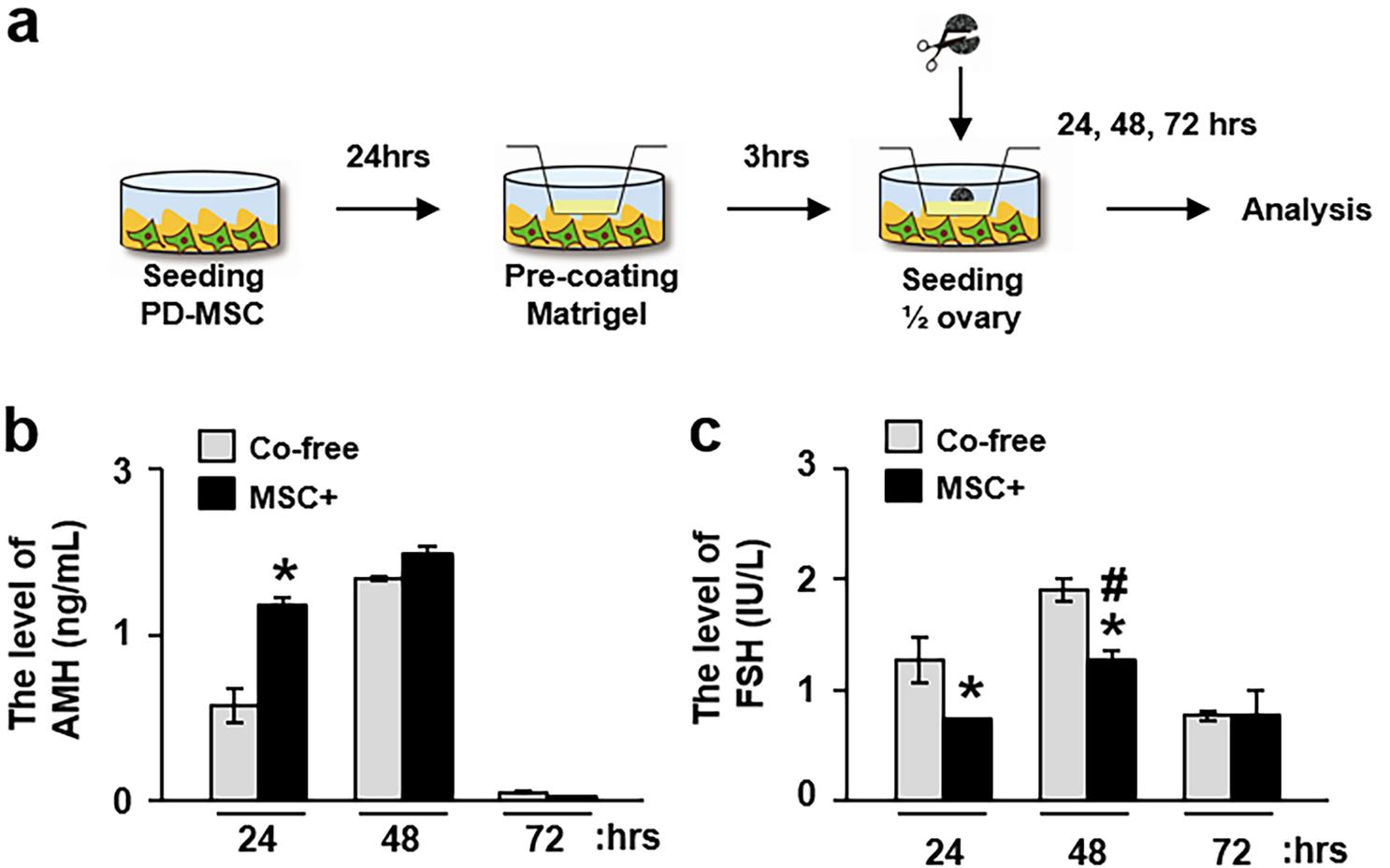


Figure 5

Serum hormone levels in the excised ovaries of rats after coculture with PD-MSCs. (a) Schematic illustration of how the ex vivo coculture system was established. One-half of the rat ovary was cultured with or without PD-MSCs for 24, 48, and 72 hours. The levels of (b) AMH and (c) FSH in the supernatants at 24, 48, and 72 hours after PD-MSC coculture were analyzed by enzyme-linked immunosorbant assay (mean \pm SE). All experiments were performed in triplicate. Significance at $p < 0.05$ is indicated by * for NTx vs. others and # for DTx vs. TTx.

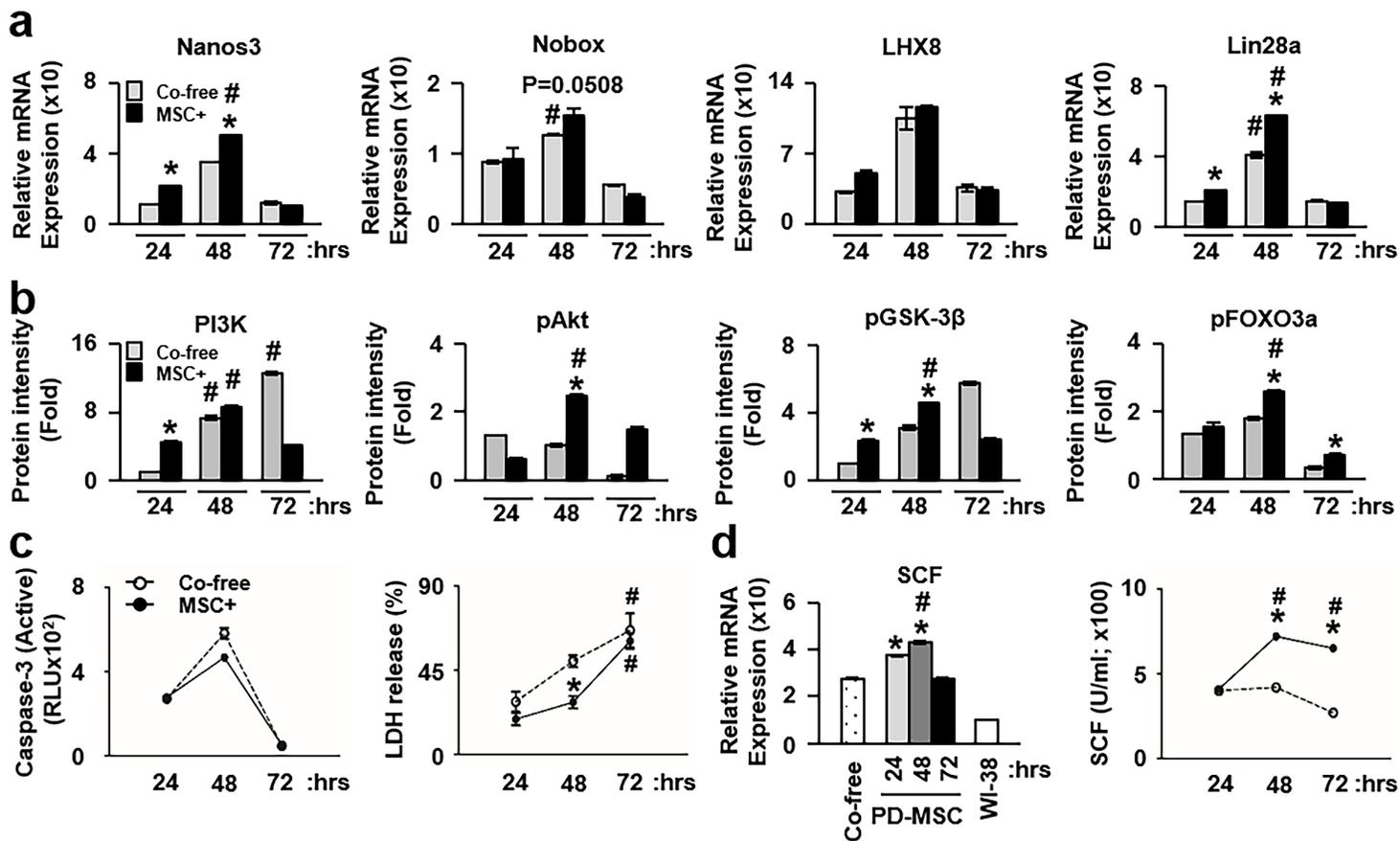


Figure 6

Expression of genes involved in ovarian function in excised rat ovaries after coculture with PD-MSCs. The mRNA expression of (a) Nanos3, Nobox, LHX8 and Lin28a in the ovaries of rats at 24, 48, and 72 hours after coculture with PD-MSCs was analyzed by quantitative real-time polymerase chain reaction, with reads normalized to GAPDH as an internal control (mean±SE). The intensity of (b) PI3K, pAkt, pGSK3β and pFOXO3a and (c) the activities of caspase-3 in supernatants and in ovary tissues at 24, 48, and 72 hours after coculture with PD-MSCs were analyzed by Western blotting and enzyme-linked immunosorbent assays (mean±SE). The loading control was GAPDH for qRT-PCR and Western blotting. The percentage of LDH release was measured by the LDH assay. (d) mRNA expression (left) and activity (right) of SCF in ovaries and supernatants at 24, 48 and 72 hours after coculture with or without PD-MSCs or WI-38 cells were analyzed by quantitative qRT-PCR and enzyme-linked immunosorbent assay, normalized to GAPDH as an internal control (mean ± S.E.). All experiments were performed in triplicate. Significance at $p < 0.05$ is indicated by * for NTx vs. others and # for DTx vs. TTx (±S.E.).

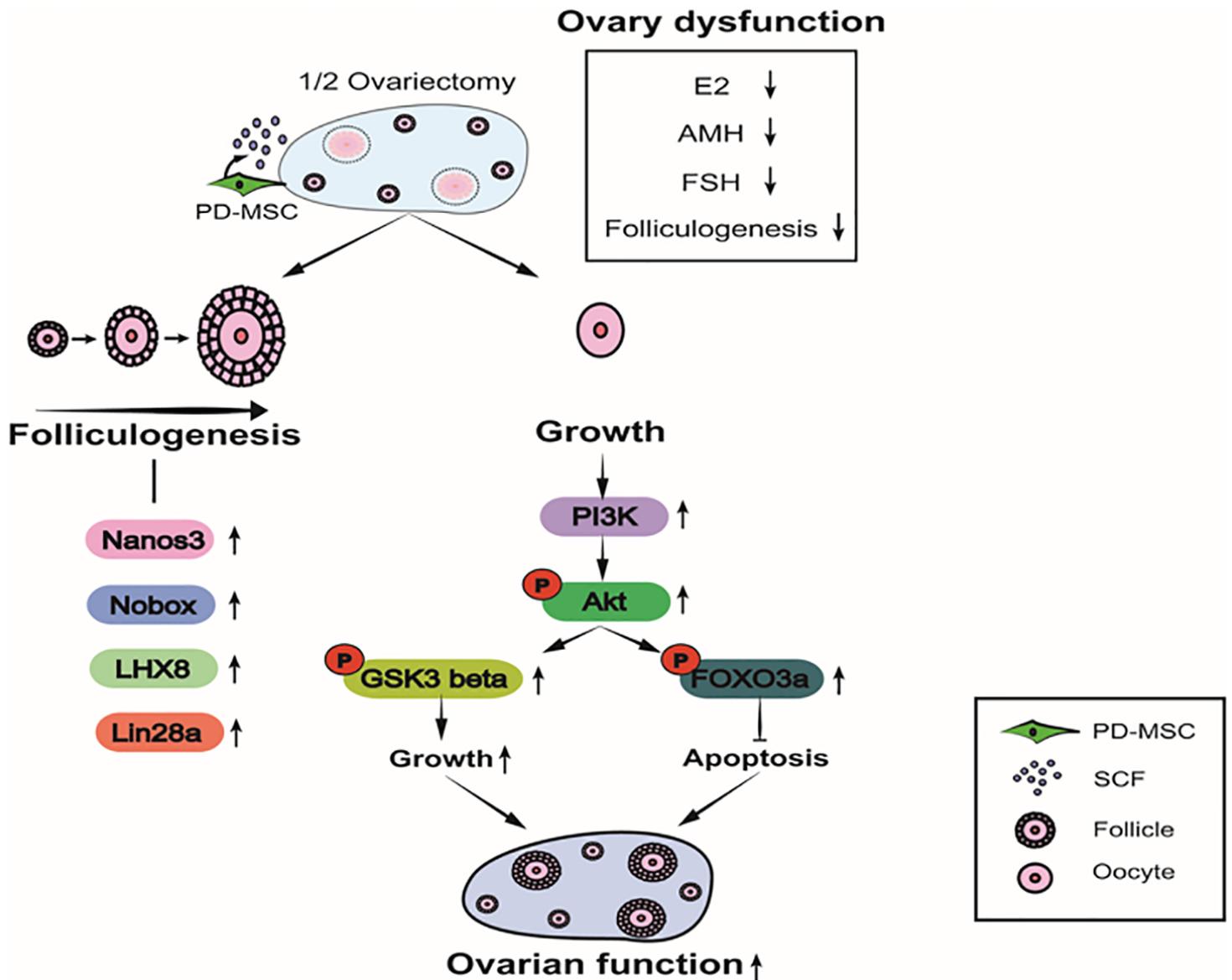


Figure 7

Summary of the ability of PD-MSCs to restore ovary function in the ovariectomized model. PD-MSCs promote folliculogenesis in the ovary, as indicated by upregulation of pathway markers, including Nanos3, Nobox, LHX8 and Lin28a. Additionally, SCF expressed in PD-MSCs dramatically increased pGSK3 β , which is involved in oocyte growth, and decreased oocyte apoptosis by inducing pFOXO3a expression via the PI3K-pAkt pathway.

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